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TECHNICAL MANUSCRIPT 36

THE EFFECT OF Ca^{++} AND Mg^{++} ON LYSIS, GROWTH, AND PRODUCTION OF VIRULENCE ANTIGENS BY PASTEURELLA PESTIS

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THE EFFECT OF CA^{++} AND MG^{++} ON LYSIS, GROWTH, AND PRODUCTION OF VIRULENCE ANTIGENS BY PASTEURELLA PESTIS

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ABSTRACT

Experiments were designed to define further the previously described phenomena of virulence antigen production, bacterial stasis, and lysis in virulent Pasteurella pestis. The observations, by Lawton, that Mg^{++} "induces" and Ca^{++} "represses" virulence antigen production were verified; optimal production of these antigens occurred in a chemically defined medium containing 0.02 M $MgCl_2$. and no added $CaCl_2$. As shown by Higuchi and others, this environment results in stasis of cells possessing the genetic potential for producing virulence antigens. The addition of 0.0025 M Ca^{++} in the presence of 0.02 M Mg^{++} completely repressed the production of these antigens and permitted cell division to occur. Cells of the rare avirulent mutant type that produces virulence antigens and forms atypical colonies on agar made Ca^{++}-deficient grow more slowly in Ca^{++}-deficient broth than do those avirulent cells that produce no virulence antigens. Under these conditions, the former type produces a lower titer of virulence antigens than do virulent cells that remain static. The generally accepted qualitative correlation between stasis and virulence antigen production has been placed on a semi-quantitative basis. A population of fully induced virulent cells remains static and almost fully viable for at least four days. Of 16 tested energy sources, none was found to enhance virulence antigen production in the presence of Mg^{++} without equally favoring cell division in the presence of Ca^{++}. Induced virulent cells retained their morphological integrity; however, they appeared to be somewhat larger and the formation of new cross-septa was not observed. In contrast, swelling followed by lysis was seen in Mg^{++}-deficient media containing sufficient Ca^{++} to repress virulence antigen production and thus initiate growth. As previously shown by Wessman and co-workers, the particular energy source employed in Mg^{++}-deficient medium was of primary importance in determining whether lysis would occur. Ca^{++} plays a minor role in preventing lysis and there is no evidence that the virulence antigens contributed to the lytic phenomenon in the media employed. The concentration of Ca^{++} required to repress virulence antigen production or permit cell division in vitro is similar to that contained in intravascular fluid. Similarly, the optimal concentrations of Mg^{++} and Ca^{++} required for induction of virulence antigens and concomitant stasis in vitro are identical to those reported for intracellular fluid.

CONTENTS

FIGURES

TABLES

I. INTRODUCTION

Burrows and co-workers have demonstrated that the phenotypic expression of virulence in Pasteurella pestis is dependent upon the presence of at least four genetic factors that may be detected in vitro. These virulence properties are the ability to (a) synthesize purines,[1] (b) produce VW or virulence antigens,[2] (c) produce capsular or Fraction I antigen,[3] and (d) form pigmented colonies on a solid synthetic medium containing hemin.[4] Mutants that fail to synthesize purines, to produce VW antigens, or to pigment exhibit a qualitative loss of virulence in the mouse or guinea pig, i.e., an increase in LD_{50} from less than ten to approximately 10^8 and 10^9 cells, respectively. Strains that lack solely the ability to produce capsular antigen exhibit a quantitative loss of lethality[3] but not necessarily of infectivity[5] in the guinea pig; virulence in the mouse is not altered.

Mutants lacking solely the ability to pigment or to synthesize purines may be restored to full virulence in the mouse by suitable injection of iron[6] or purine,[1] respectively. Accordingly, strains of these types are frequently termed potentially virulent in distinction from the outright avirulent type that has lost the ability to produce detectable amounts of virulence antigens (VW). At present, this latter type cannot be restored to virulence by specific manipulation of any known host species. Thus, only the loss of the VW determinant results in both an irreversible and qualitative loss of virulence. These observations indicate that the properties of pigmentation, as well as the ability to produce capsular antigen and purines, may be lost without loss of virulence in the suitably treated mouse. Presumably, these three properties are necessary for plague outbreaks in nature; however, isolation of a capsular antigen-deficient strain from a case of plague has been reported.[7] On the other hand, possession of the VW determinant seems to be essential for survival in nature as well as for virulence in the laboratory.

Wessman et al[8] demonstrated that cells of five virulent nonglycerol fermenting strains lyse at 37°C when placed in a chemically defined glucose medium containing 10^{-4} M Ca^{++} and 0.002 M Mg^{++}. Lysis could be prevented by lowering the temperature of incubation to 36°C or by increasing the concentration of Mg^{++} to 0.022 M. No lysis was observed in the case of seven avirulent strains that were grown under similar conditions. Subsequently, Higuchi et al[9] reported that one potentially virulent and nine virulent strains required 0.002 to 0.004 M Ca^{++} in order to grow aerobically at 37°C in a synthetic medium containing 0.02 M Mg^{++}; no requirement for Ca^{++} was observed when five avirulent strains were tested. Higuchi and Smith[10] later developed an agar medium containing added Mg^{++} and oxalate ions that is selective for avirulent cells that were found to arise at a mutation rate of 10^{-4}. It was also shown, by various workers, that this avirulent mutant type, which is able to grow at 37°C at high Mg^{++} levels in the absence of Ca^{++}, no longer produces VW.[11,12]

However, the concomitant loss of VW and Ca^{++} dependence does not always occur, since Brubaker and Surgalla[13] isolated avirulent strains that do not require Ca^{++} for growth at 37°C but still produce VW. These strains were termed VW^{+} avirulent mutants in contrast to the more common VW^{-} avirulent type. Subsequently,[14] the growth of a virulent, a VW^{+} avirulent, and a VW^{-} avirulent strain was studied in broth made deficient in Ca^{++}. At 37°C, the growth rate of the VW^{+} avirulent mutant was intermediate between that of the rapidly dividing VW^{-} avirulent mutant and that of the virulent parent, which essentially remained static.

This report gives additional comparative data concerning the nutrition and immunology of the virulent, VW^{-} avirulent, and VW^{+} avirulent genotypes. Emphasis is placed on the glucose effect of Wessman[8] and previously noted effects concerning the stimulatory effect of Mg^{++} and inhibitory effect of Ca^{++} on VW production.[16,11]

II. MATERIALS AND METHODS

Stock cultures, methods of preparing inocula, and storage procedures have been described previously.[14] Difco blood agar base (BAB), BAB plus 0.01 M $CaCl_2$ (BABC), and the magnesium oxalate agar (MGOX) of Higuchi and Smith[10] were employed as plating media. Difco heart infusion broth (HIB) was used in the majority of experiments concerning growth, cellular morphology, and antigen production. Solutions of $CaCl_2$, $MgCl_2$, Na oxalate, and the various energy sources employed were sterilized separately and added aseptically to flasks containing sterile HIB. Optical density was determined at 600 millimicrons on a Coleman Model 9 nephlo-colorimeter; a reading of 0.050 corresponds to 1.08×10^8 cells per milliliter of medium. Antigen analyses were performed by the gel-diffusion procedure employed by Lawton *et al*;[16,17] samples from whole cultures were used as the source of antigen. The antigenic nomenclature employed by these workers was used in this investigation. Antisera were kindly provided by Dr. W. D. Lawton.

The basal synthetic medium (Table I) employed in experiments concerned with lysis is a modification of that developed by Higuchi *et al*.[9] The amino acids, less tryptophan and cysteine-HCl, were prepared as a double-strength solution. Citric acid, K_2HPO_4, $FeSO_4$, and $MnSO_4$ were prepared as a tenfold-strength stock salt solution. Tryptophan (dissolved in a minimal amount of NaOH) and cysteine-HCl were brought into solution separately, added with the vitamins and sterilized by filtration as a fivefold-strength stock solution. Solutions of the various energy sources under study were prepared at 50- or 100-fold concentration and also sterilized by filtration except for Na fumarate, which was autoclaved directly in the medium. $CaCl_2$, $MgCl_2$, and $Na_2S_2O_3$ were prepared at 100-fold strength and sterilized by autoclaving. The complete medium was prepared by adding suitable quantities of the amino acid and salt solutions into Erlenmeyer flasks fitted with cotton stoppers. Phenol red (10 ppm) was added, the flasks were sterilized by autoclaving, brought to pH 6.8 to 6.9 with 5N NaOH, and then appropriate amounts of the sterile tryptophan-cysteine-vitamin, Mg^{++}, Ca^{++}, $Na_2S_2O_3$, and energy source solutions were added aseptically; sufficient sterile distilled water was added so that the medium was brought to volume upon addition of the inoculum. When the energy sources were mono-sodium salts or carbohydrates, additional NaCl was added so that the final concentration of Na^+ in the medium was identical to that obtained when di-sodium salts were employed. Inocula were prepared from cells previously grown at 37°C in the above medium containing 0.02 M Mg^{++} and 0.002 M Ca^{++}. The cells were harvested in the late log phase, washed in 0.033 M potassium phosphate buffer, pH 7.2, and finally resuspended at suitable concentration in distilled water.

Reagent grade salts were employed in all experiments; vitamins and amino acids were obtained from the Nutritional Biochemical Corporation, Cleveland, Ohio. Flasks were aerated by shaking on a reciprocating shaker with a 2 3/4-inch stroke at 93 excursions per minute.

TABLE I. BASAL MEDIUM FOR STUDY OF THE EFFECT OF CA^{++} AND MG^{++} ON GROWTH, LYSIS, AND W ANTIGEN PRODUCTION

Amino Acids	Millimoles per Liter	Vitamins	Micromoles per Liter
Glycine	10.0	Thiamin.HCl	5.0
DL-Alanine	2.0	Ca pantothenate	2.0
L-Serine	1.0	Biotin	2.0
DL-Cysteine.HCl	2.0		
DL-Threonine	2.0		
L-Methionine	2.0		
L-Valine	2.5	Salts [a]	mM/L
L-Leucine	1.0	Citric Acid	10.0
DL-Isoleucine	2.0	K_2HPO_4	25.0
L-Aspartic Acid	2.5	$FeSO_4$	0.1
L-Glutamic	15.0	$MnSO_4$	0.01
L-Lysine.HCl	1.0	$Na_2S_2O_3$	2.5
L-Proline	5.0		
L-Hydroxyproline	1.0		
L-Histidine.HCl	1.0		
L-Trystophan	1.0		
L-Arginine.HCl	1.5		
DL-Phenylalanine	5.0		
L-Tyrosine	1.0		

a. $MgCl_2$, $CaCl_2$, and NaCl as well as various energy sources were added as indicated in the text.

III. RESULTS

The initial growth of strain MP6 and its VW⁻ avirulent mutant, strain OX/MP6, was determined turbidimetrically at 37°C in HIB (adjusted to pH 6.8 with 5N HCl) containing 0.25 per cent glucose plus various combinations of Ca^{++} (0.01 M), Mg^{++} (0.02 M), and Na oxalate (0.02 M). After incubation for six hours, the pH of those cultures that supported cell division was approximately 5.5; at this time the experiment was terminated.

As shown in Figure 1, the initial growth rate of the VW⁻ avirulent strain OX/MP6 was not significantly affected by any of the salts employed; however, production of inhibitory concentrations of acid in those avirulent cultures containing Mg^{++} was delayed, thus resulting in a slightly greater terminal turbidity. Comparable growth of the virulent strain MP6 was obtained in the presence of Ca^{++} (Figure 2). In these experiments, the sole addition of oxalate ions did not alter the growth rate over that of the control; however, massive cell lysis was often observed upon continued incubation. The addition of either Mg^{++} or Mg^{++} plus oxalate ions to virulent cultures resulted in the stasis reported by Higuchi and co-workers;[9] only partial inhibition was observed in the presence of Mg^{++} plus Ca^{++}.

Bacterial stasis was further studied in long-term experiments in which differential viable counts were made on BABC and MGOX. Galactose was substituted for glucose since, as observed by Ross, Hokes, and Herbert, acid production from this hexose is less than is the case with glucose.[4] Either Mg^{++} (0.02 M) and Na oxalate (0.02 M) or Ca^{++} (0.01 M) was added to 100 milliliters of HIB plus galactose (0.25 per cent) contained in two liter flasks, which were inoculated with approximately 10^8 Ca^{++} and galactose-adapted cells per milliliter of fresh medium. The flasks were aerated at 37°C for four days, during which time the pH was maintained near neutrality. The observed growth and population changes are shown in Table II. Virulent cells remained viable but static in the presence of Mg^{++} and oxalate ions and were overgrown by the avirulent population after one day. The latter entered a precipitous death phase after two days and by the fourth day the surviving virulent population was again in predominance at a cell concentration of approximately half of that contained in the original inoculum. As expected, the number of avirulent cells in the virulent culture that received added Ca^{++} never exceeded the virulent population. No significant difference was observed between the virulent and VW⁻ avirulent cultures that contained added Ca^{++}; however, the stationary phase was prolonged by one day in the case of the avirulent culture that received added Mg^{++} and oxalate ions.

As well as verifying the results obtained by Higuchi *et al*,[9] these experiments indicate that cell division of the virulent strain under these conditions is immediately suppressed in 0.02 M Mg^{++} in the absence of Ca^{++}, and that such suppressed cells may remain static but almost fully viable for at least four days.

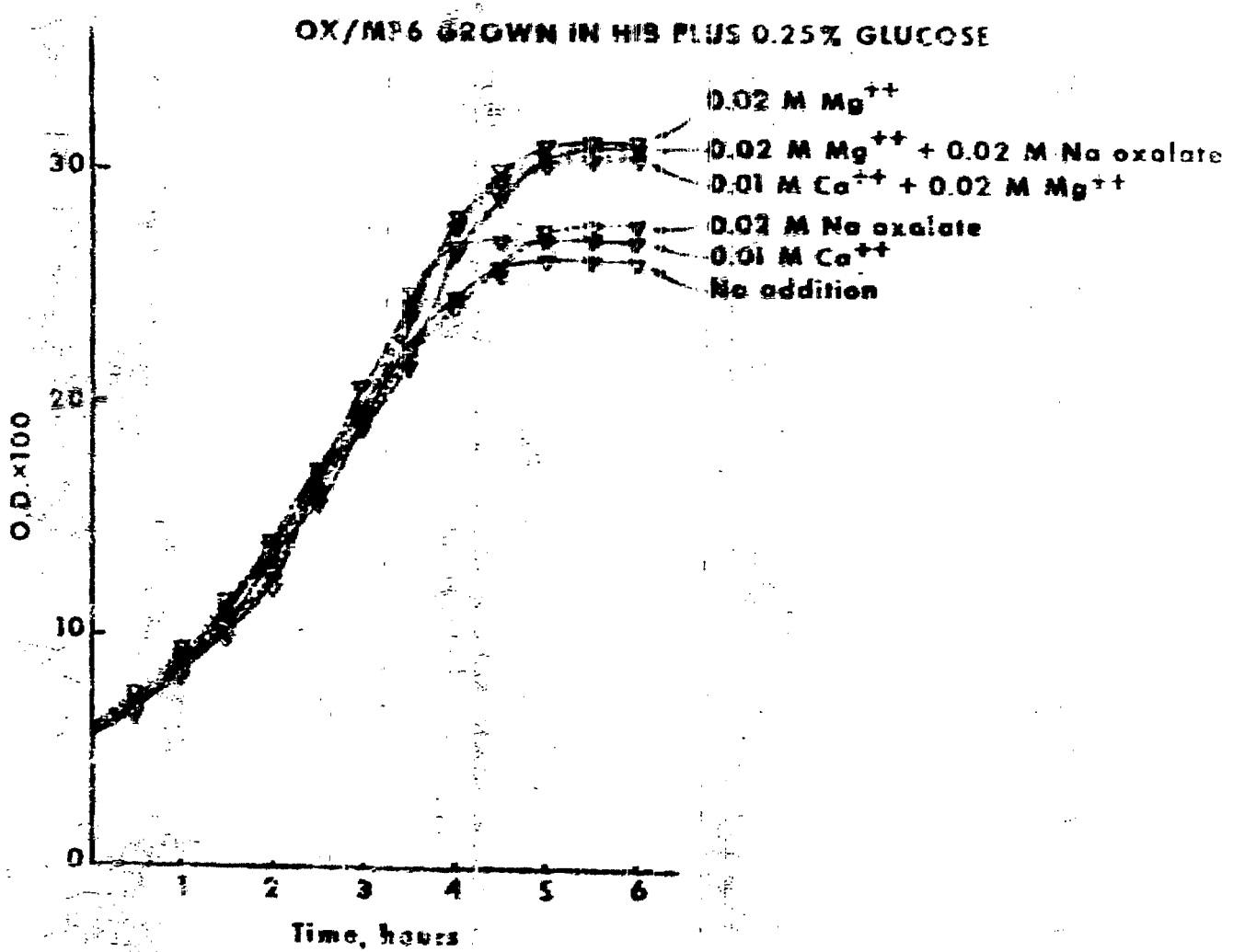

Figure 1. Inability of Ca^{++} or Mg^{++} to Affect the Initiation of Growth of a 'W' Avirulent Strain of P. mirabilis.

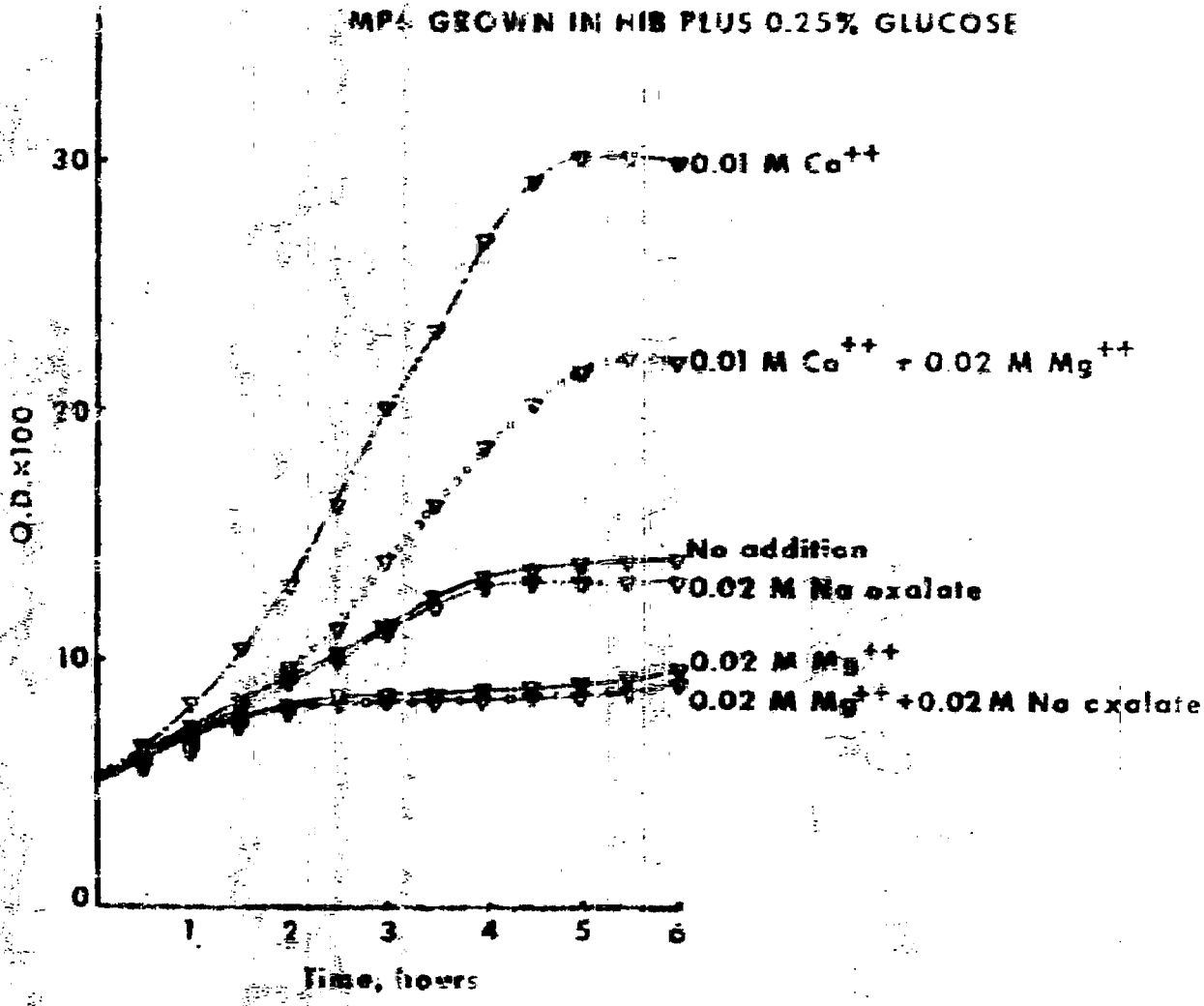

Figure 2. Effect of Ca^{++} and Mg^{++} on the Initiation of Growth of a Virulent Strain of P. pestis.

TABLE II. TOTAL AND AVIRULENT VIABLE COUNTS OF STRAINS MP6 AND OX/MP6[a]

MEDIUM SUPPLEMENT	TIME OF INCUBATION, days	MP6 Viable counts		OX/MP6 Viable counts	
		BABC	MGOX	BABC	MGOX
Ca^{++}	1	4.2×10^{9}	2.0×10^{6}	4.5×10^{9}	4.8×10^{9}
	2	1.1×10^{9}	1.2×10^{6}	9.7×10^{8}	8.2×10^{8}
	3	8.6×10^{7}	4.2×10^{5}	1.8×10^{8}	1.5×10^{8}
	4	4.2×10^{7}	1.4×10^{5}	4.7×10^{7}	3.8×10^{7}
Mg^{++} and	1	2.3×10^{8}	5.0×10^{7}	4.2×10^{9}	4.1×10^{9}
Na oxalate	2	1.4×10^{9}	1.5×10^{9}	3.0×10^{9}	3.3×10^{9}
	3	3.8×10^{8}	3.3×10^{8}	1.2×10^{9}	9.8×10^{8}
	4	8.5×10^{7}	4.6×10^{6}	1.8×10^{8}	1.5×10^{8}
Original inoculum		1.8×10^{8}	7.4×10^{4}	2.1×10^{8}	2.0×10^{8}

a. Incubated at 37°C in HIB plus 0.25 per cent galactose and either 0.01 M Ca^{++} or 0.02 M Mg^{++} plus 0.02 M Na oxalate.

Growth of the VW^{+} avirulent type (not shown) was rapid in the presence of Ca^{++} and somewhat slower than that of the VW^{-} avirulent strain in the presence of 0.02 M Mg^{++} and oxalate ions.[14]

Cells in virulent cultures frequently lysed after 10 to 15 hours when grown in the presence of oxalate ions. This effect was completely suppressed by the addition of 0.02 M Mg^{++}, thus suggesting a similarity to the glucose effect of Wessman.[8] Lysis was never observed in VW^{+} or VW^{-} avirulent cells inoculated without galactose.

The morphology of virulent cells grown or suspended in HIB containing galactose plus various concentrations of Mg^{++} and Ca^{++} was examined by phase contrast microscopy. Rapidly growing cells obtained from media containing added 0.01 M Ca^{++} were typical small bipolar rods in which newly forming cross-septa were usually apparent. When these cells were centrifuged, washed, and resuspended in medium containing added 0.02 M Mg^{++} and 0.02 M Na oxalate, cell division rapidly ceased and formation of new cross-well material was not observed. After subsequent incubation for 12 to 18 hours, numerous diplobacilli were seen; these presumably arose from cells that were about to divide when removed from the Ca^{++}-rich growth medium. Attempts to demonstrate the presence of cross-septa within these diplobacilli by suspension in saline at various tonicities were not successful.[18] Upon suitable transfer to media

containing Ca^{++}, it was possible to observe the formation of new cross-septa followed by partially synchronized cell division. If the virulent cells were maintained in Ca^{++}-deficient media for more than 24 hours, rapidly dividing types became visible at a time that corresponded to the overgrowth of the culture by the avirulent population. When the virulent cells were suspended in media containing oxalate ions and 0.01 M or less Mg^{++}, an increase in size was usually observed. If oxalate ions but no Mg^{++} were employed, the cells always became greatly enlarged and frequently lysed; during this phase bizarre forms and spheroplasts were sometimes observed. These morphological changes were not observed in VW^{+} or VW^{-} avirulent cells tested under identical conditions. The morphological differences between virulent and VW^{-} avirulent cells after 12 hours' incubation in HIB plus 0.25 per cent galactose, 0.01 M Mg^{++}, and 0.02 M Na oxalate are illustrated in Figure 3.

With sufficient data concerning the effect of Ca^{++} and Mg^{++} on growth in HIB, we were in a position to relate these effects to production of antigens. Lawton[15] had demonstrated that these cations markedly affect the production of VW in virulent strains; however, their role in the production of other antigens, and in production of the virulence antigens by VW^{+} avirulent strains, was not known. In these comparative studies, the virulent strain Siam and its VW^{+} avirulent (strain VW/Siam) and VW^{-} avirulent mutant (strain OX/Siam) were employed, as well as six additional virulent strains and their VW^{+} avirulent mutants.

Production of antigens E, F (capsular antigen), I (antigen 4 of Crumpton and Davies[19]) K, L, Q, T (murine toxin), V and W was semi-quantitatively determined.[17] Antigen E is oxygen- and partially temperature-dependent; this antigen was employed as a control, because it was believed to be produced with equal facility by both virulent and avirulent cells. The above strains were inoculated at 10^{8} cells per milliliter into 500-milliliter flasks containing 50 milliliters of HIB plus 0.03 M glucose and either 0.01 M Ca^{++} or 0.02 M Mg^{++} and 0.02 M Na oxalate; phenol red (10 ppm) was added to facilitate control of pH. The flasks were incubated for 12 hours, during which time the pH was maintained near neutrality by addition of 1N NaOH.

The units of various antigens detected per 10^{9} viable cells are recorded in Table III. The titers of antigen W, and to a lesser degree of antigen V, but not of the remaining antigens, are increased in the two genetically competent strains by the addition of Mg^{++} and oxalate ions. In order to verify that the production of VW is favored by Mg^{++} and not by a state of Ca^{++} deficiency, the above experiment was repeated employing Mg^{++} (0.02 M) in various combinations with Ca^{++} (0.01 M) and Na oxalate (0.02 M) controls. The results of this experiment, including differential viable counts and determinations of antigen E and W, are shown in Table IV. Only minor differences in production of antigen E were observed; however, the sole addition of Mg^{++} greatly favored the synthesis of antigen W.

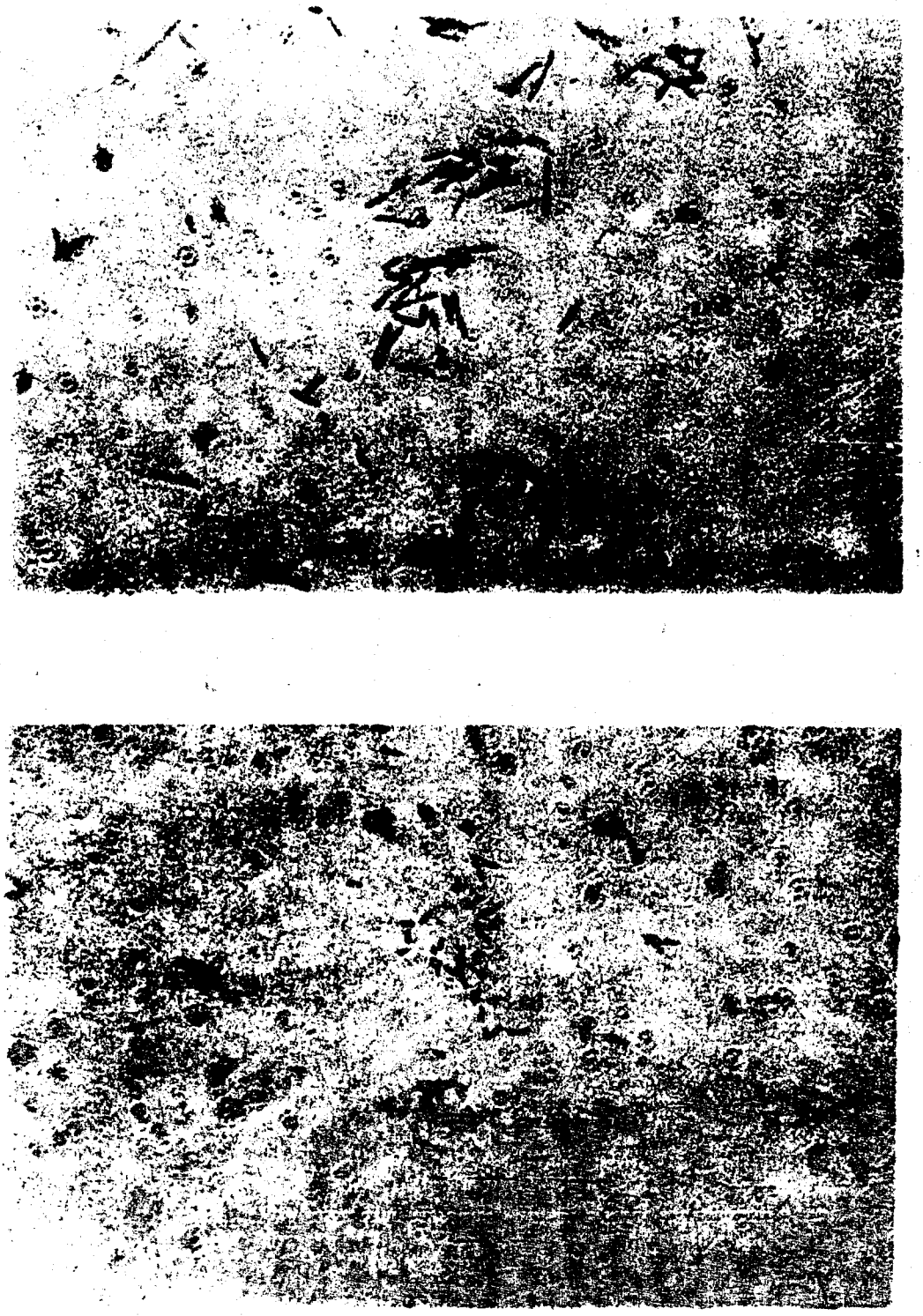

Figure 3. TOP: Enlarged Virulent Cells Viewed by the Phase Contrast Microscope after Incubation for 12 hours in Ca^{++} -deficient Medium Containing 0.01 M Mg^{++}. BOTTOM: VW- Avirulent Cells Exhibiting Normal Morphology following Identical Treatment.

TABLE III. EFFECTS OF CALCIUM AND MAGNESIUM OXALATE ON PRODUCTION OF VARIOUS ANTIGENS [a]

TEST ANTIGEN	Units Antigen Per 10^9 Cells					
	Siam		VW/Siam		OX/Siam	
	Ca^{++} [b]	Mg^{++},OX [b]	Ca^{++}	Mg^{++},OX	Ca^{++}	Mg^{++},OX
E	1	2	1	1	9	8
F	15	18	4	4	19	16
I	0	0	0	0	1	1
K	30	36	34	30	38	32
L	1	2	1	1	2	2
Q	7	9	0	2	10	4
T	7	9	4	4	10	8
V	1	9	1	4	0	0
W	0	36	0	17	0	0

a. At 37°C upon incubation for 12 hours in HIB plus 0.03 M glucose inoculated at 10^8 cells per milliliter.
b. Additions to HIB.

TABLE IV. ANTIGEN E AND W PRODUCTION BY A VIRULENT AND TWO RELATED AVIRULENT STRAINS OF P. PESTIS GROWN FOR 12 HOURS IN HEART INFUSION BROTH CONTAINING 0.03 M GLUCOSE

ADDITION TO PRODUCTION MEDIUM	VIABLE COUNTS[a]		ANTIGEN TITERS[b]		UNITS/10^9 CELLS	
	Total	Avirulent	E	W	E	W
	Strain Siam (virulent)					
None	4.8×10^8	2.4×10^5	1:2	0	4	0
0.01 M Ca^{++}	1.1×10^9	2.8×10^5	1:2	0	2	0
0.02 M Na oxalate	9.4×10^6	3.7×10^3	1:2	0	-	-
0.02 M Mg^{++}	4.9×10^8	2.9×10^6	1:2	1:32	4	65
0.02 M Mg^{++} plus 0.02 M Na oxalate	4.5×10^8	2.5×10^5	1:2	1:8	4	18
0.02 M Mg^{++} plus 0.01 M Ca^{++}	1.2×10^9	3.6×10^5	1:8	0	7	0
original inoculum	2.3×10^8	8.7×10^4	0	0	0	0
	Strain VW/Siam (avirulent)					
None	3.0×10^8	3.0×10^8	1:1	0	3	0
0.01 M Ca^{++}	9.5×10^8	9.6×10^8	1:1	0	1	0
0.02 M Na oxalate	2.4×10^8	2.4×10^8	1:1	1:2	4	8
0.02 M Mg^{++}	5.6×10^8	5.7×10^8	1:1	1:16	2	29
0.02 M Mg^{++} plus 0.02 M Na oxalate	5.2×10^8	5.1×10^8	1:1	1:4	2	8
0.02 M Mg^{++} plus 0.01 M Ca^{++}	5.4×10^8	5.3×10^8	1:1	0	2	0
original inoculum	1.5×10^8	1.5×10^8	0	0	0	0
	Strain OX/Siam (avirulent)					
None	2.8×10^8	2.9×10^8	1:2	0	7	0
0.01 M Ca^{++}	8.5×10^8	8.4×10^8	1:4	-	5	0
0.02 M Na oxalate	6.6×10^8	6.5×10^8	1:4	0	6	0
0.02 M Mg^{++}	8.8×10^8	8.7×10^8	1:4	0	5	0
0.02 M Mg^{++} plus 0.02 M Na oxalate	1.0×10^9	1.0×10^9	1:8	0	8	0
0.02 M Mg^{++} plus 0.01 M Ca^{++}	8.1×10^8	8.1×10^8	1:8	0	10	0
original inoculum	1.5×10^8	1.4×10^8	0	0	0	0

a. Total count determined on BAB plus 0.01 M Ca^{++}; avirulent count determined on magnesium oxalate agar.

b. Greatest dilution of antigen per 1.0 ml that exhibits a band of precipitate when tested under standard conditions.

The addition of Ca^{++} in the presence of Mg^{++} resulted in complete suppression of W antigen, whereas the sole addition of oxalate ions resulted in lysis (Strain Siam) or partial suppression of W antigen production (Strain VW/Siam). Identical results were obtained with the other tested virulent strains and their VW^{+} avirulent mutants. It is significant that W antigen production by the VW^{+} avirulent strains was always about half of that observed for their virulent parents. The only VW^{+} avirulent mutant that was examined for production of antigens, other than V and W, was Strain VW/Siam, which exhibited apparent deficiency in antigen F, Q, and T production.

Gluconate has recently been shown to favor virulence antigen production.[15, 20] Investigation of the metabolism of gluconate by Mortlock[21] has resulted in the detection of all of the enzymes of both the Entner-Douderoff and hexose-monophosphate-oxidative pathways with the notable exception of glucose-6-phosphate dehydrogenase.[22] This finding might be correlated with the observation that gluconate and ribose, but not glucose, stimulated the production of V antigen in the test system employed by Lawton.[15] In order to test the hypothesis that gluconate and metabolically related energy sources are more active inducers of virulence antigen synthesis than are hexoses or other compounds such as permeable Kreb's cycle intermediates, the effect on W antigen synthesis of 16 energy sources was tested in HIB containing 0.02 M Mg^{++}. It was found in these experiments that if the pH was maintained at neutrality by addition of 1N NaOH, the use of glucose, fructose, or mannose stimulated greater production of W antigen than did gluconate, ribose, or xylose. Relatively little W antigen was produced in the presence of pyruvate or the four tested Kreb's cycle intermediates.

An experiment was designed to determine quantitatively the above relationship, since those energy sources known to support good growth of virulent cells in the presence of Ca^{++} were also those that stimulated greater W antigen production in the presence of Mg^{++}. Growth from a BAB slope of Strain Siam was inoculated into 100 milliliters of HIB plus 0.03 M glucose and 0.01 M Ca^{++} contained in a two-liter flask. This culture was serated by shaking at 37°C until the cells approached the stationary phase, at which time they were removed by centrifugation, washed in sterile HIB, and inoculated at a concentration of 10^5 cells per milliliter into 50 milliliters of HIB plus 0.01 M Ca^{++} and 0.03 M energy source contained in 500-milliliter flasks. The cultures were aerated for 12 hours at 37°C, at which time viable counts were performed on BABC and on MGOX. W antigen was detected at low titer only in the flask containing gluconate. Subsequently, W antigen production in HIB plus 0.02 M Mg^{++}, 0.02 M Na oxalate, and 0.03 M energy source was determined by inoculating at 10^8 cells per milliliter and aerating the cultures for 18 hours at 37°C. Oxalate ions were added to the medium to prevent significant multiplication of the virulent cells during incubation. For each energy source tested, the number of bacterial generations obtained in the medium containing Ca^{++} is plotted in Figure 4 against the units of W antigen produced in medium containing Mg^{++} and oxalate ions. It is clear that there is no tested energy source that

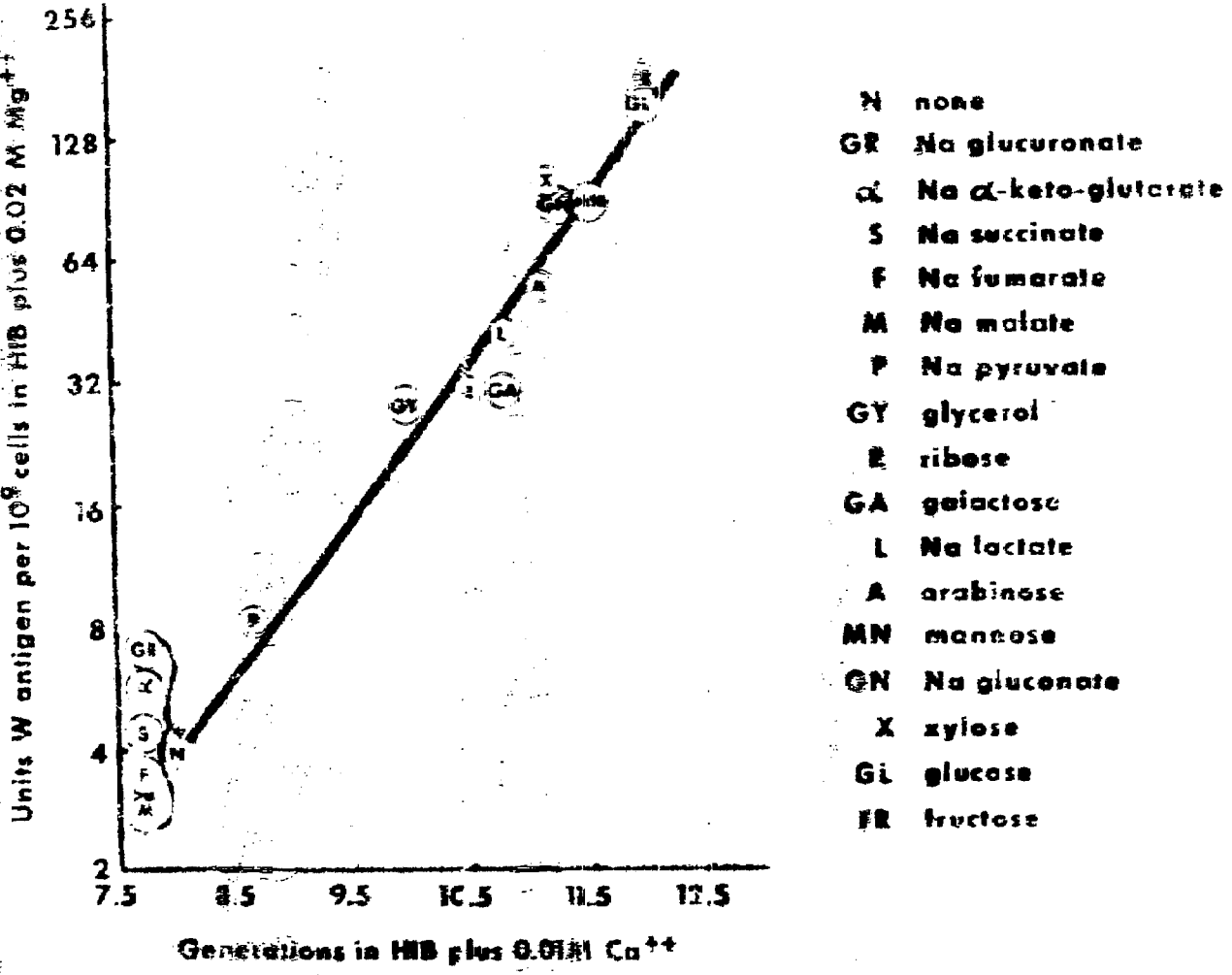

Figure 4. Relationship between W antigen Production in the Presence of Mg^{++} and Growth in the Presence of Ca^{++} as a Function of the Energy Source Supplied.

stimulates the production of W antigen in the presence of added Mg^{++} without equally favoring bacterial multiplication in the presence of added Ca^{++}.

From the work of Wessman, Lawton, Higuchi, and that reported above, it is apparent that in media deficient in Ca^{++} (but otherwise sufficient to support growth at 37°C) the addition of Mg^{++} prevents lysis and induces VW production, but does not permit growth of virulent cells. In this study, lysis at 37°C of virulent cells suspended in HIB containing added oxalate ions was erratic. This variability might be associated with endogenous Mg^{++}, a cation not easily removed from HIB without untoward chemical change or loss of other constituents. Accordingly, the synthetic medium of Higuchi et al[9] was modified so that VW was produced at titers comparable with that observed in HIB. The medium used by the above workers differs from the modification employed in these experiments in that we (a) altered the concentration of amino acids employed and included serine, aspartic acid, histidine, and hydroxyproline; (b) eliminated NH_4 acetate because we found that acetate ions (and formate ions) may be toxic at 37°C; and (c) increased the Na gluconate concentration to 0.03 M and eliminated xylose. This medium was designed primarily to facilitate protein synthesis; it does not represent the minimal nutritional requirements for VW production. Na gluconate was employed as an energy source, since the utilization of this substance results in only minor changes of pH.

Growth form Strain Siam was inoculated at a concentration of 1.7×10^8 cells per milliliter into 250-milliliter flasks containing 12.5 milliliters of synthetic medium at 0.0003 to 0.005 M Ca^{++} and 0.0012 to 0.04 M Mg^{++}. Growth, cellular morphology, and W antigen production were determined after incubation at 37°C for 15 hours; the results are illustrated in the model in Figure 5. Massive lysis (viable counts less than 10^6) occurred at 0.0012 M Mg^{++} regardless of the concentration of Ca^{++} employed. However, no lysis occurred at increased twofold to fourfold concentrations of Mg^{++} provided that at least 0.0025 M Ca^{++} was present. Neither significant growth nor lysis occurred at low concentrations of Ca^{++} (0.0003 to 0.0012 M) in the presence of high concentrations of Mg^{++} (0.01 to 0.04 M); however, cells placed in the presence of low amounts of Ca^{++} and 0.01 M Mg^{++} exhibited a definite increase in size. At least 0.0025 M Ca^{++} was required for growth and little or no W antigen was detected in flasks that contained this or the higher concentration employed. W antigen titers of approximately 60 units per 10^9 cells were obtained at Ca^{++} concentrations of 0.0012 M or less, provided that at least 0.01 M Mg^{++} was present.

These results differ slightly from those reported by Higuchi, who detected growth rather than lysis of virulent cells at 0.0025 M Mg^{++} in the absence of Ca^{++}. This discrepancy may be associated with the altered amino acid content or the deletion of xylose.

Figure 5. Model Illustrating the Role of Ca^{++} in Stimulating Growth and of Mg^{++} in Preventing Lysis in Synthetic Medium Inoculated with 1.7×10^8 Virulent Cells per Milliliter.

Substitution of 0.03 M concentrations of other energy sources for gluconate in synthetic medium containing 0.0025 M Mg^{++} and no Ca^{++} yielded results in accord with those of Wessman et al[8] and Brownlow and Wessman.[23] The experimental procedure was identical to that described above. After 15 hours' incubation at 37°C, neither lysis nor significant growth occurred in flasks containing xylose, arabinose, α-ketoglutarate, succinate, formate, or no added energy source. However, massive lysis occurred following bacterial growth of approximately two to three generations in flasks containing glucose, fructose, mannose, and gluconate. Erratic results were obtained with ribose, galactose, pyruvate, and lactate. Subsequently, it was found that xylose did not initiate growth of virulent cells in this medium containing 0.0025 M Ca^{++} unless at least 0.01 M Mg^{++} was added.

IV. DISCUSSION

A qualitative correlation exists between bacterial stasis and VW production, as evidenced by the fact that all reported Ca^{++} independent strains are avirulent and, of these, the great majority fail to produce detectable VW. This relationship was upset by the discovery of the rare VW^+ avirulent phenotype that forms rough atypical colonies on MGOX agar and produces both V and W antigens. At 37°C, the growth rate of VW^+ avirulent mutants is similar to that of virulent or VW^- avirulent mutants in medium containing sufficient Ca^{++}. However, in broth containing both Mg^{++} and oxalate ions, growth of the VW^+ avirulent mutant is intermediate between that of the typical VW^- avirulent mutant and that of the virulent parent, which remains static.[14] This study demonstrated that, under identical conditions of Mg^{++} excess and Ca^{++} deficiency, the production of VW by the VW^+ avirulent strain is again intermediate between that of the virulent prototroph and that of its presumed genetically incompetent VW^- avirulent mutant. Thus, the relationship between stasis and VW production is now on a quantitative basis and we can conclude that the production of VW *in vitro* is incompatible with cell division.

Ca^{++} may merely play a role as suppressor of VW synthesis. However, a possible function as a co-factor or integral part of the cell can not be dismissed. The latter suggestion seems improbable considering the high ($\sim$ 0.0025 M) concentration that is required for growth. Besides its normal role as a co-factor, Mg^{++} acts as an inducer of VW synthesis when present at high ($\sim$ 0.02 M) concentration. There is always the possibility that a new energy source may prove to be a specific inducer of VW; however, in view of the results obtained with the 16 tested energy sources, this seems unlikely. The previously held opinion that gluconate might play a unique role in stimulating VW production may be explained by the well-known chelating properties of this substance.

The energy source employed does seem to determine whether virulent cells will lyse in media deficient in both Mg^{++} and Ca^{++}. For example, lysis occurred following some growth in those cultures that contained the tested hexoses, and this phenomenon is undoubtedly a reflection of the glucose effect of Wessman *et al.*[8] These workers did not observe lysis when xylose was employed as an energy source, and protection against lysis in the presence of glucose was afforded by succinate, fumarate, or malate;[11] a similar protection was noted by Brownlow and Wessman[23] in the case of α-ketoglutarate. Our data verify these observations; however, in these experiments little or no growth occurred when xylose or the above organic acids were tested. Since neither significant cell division nor lysis occurs under the above conditions in the absence of any energy source, it is certain that lysis would not occur if an added energy source could not be metabolized under conditions of Mg^{++} deficiency. This explanation is probably correct in the case of a highly purified xylose preparation that did not initiate growth in the presence of Ca^{++}, or favor W antigen production in its absence, unless at least 0.01 M Mg^{++} was present. It is not known if those carbohydrates that do not induce lysis require a greater concentration of Mg^{++} for their uptake or subsequent immediate metabolism than do those energy sources that permit lysis to occur.

Wessman et al[8] reported that protection against lysis due to glucose was also afforded by very low levels of Mn^{++}, 0.00022 M. The present work provides no new information on this cation.

Presumably, other substances will be found that postpone or prevent lysis at low concentrations of Mg^{++}. For example, certain aliphatic polyamines studied by Mager[24] are known to protect spheroplasts of Escherichia coli, which can also be stabilized by Mg^{++} and Ca^{++}.[25] In the case of P. pestis, Mg^{++} deficiency may directly result in spheroplast formation followed by lysis, depending upon the constituents of the medium. Alternatively, an independent metabolic lesion concerning cell wall metabolism may exist that is lethal in the absence of sufficient Mg^{++}.

No VW was detected in lysates of virulent cultures; however, lysis may have occurred before detectable amounts of these antigens had accumulated. Since high amounts of Ca^{++} are known to suppress VW production, but fail to prevent lysis in this medium (in the presence of 0.0012 M Mg^{++}), it seems unlikely that VW was produced under these conditions or that these antigens contributed to cell lysis. However, as noted above, when sufficient Mg^{++} is added to Ca^{++}-deficient medium to stabilize virulent cells, VW is produced with concomitant stasis. These experiments verify the observations of Wessman and co-workers[8] that lysis is primarily a function of the compound supplied as energy source as well as the concentration of Mg^{++} contained in the medium.

It should be noted that the Ca^{++} requirement of Azotobacter vinelandii is somewhat similar to that observed in P. pestis. Deficiency in the former causes an increase in cellular size and postpones the initiation of growth; marked deficiency results in swollen and bizarre cells accompanied by lysis.[26] In contrast, Renaux[27] stated that Ca^{++} retards the growth of virulent Bacillus anthracis, which upon subsequent passage on Ca-rich agar, become asporogenic and attenuated. These changes could be prevented in the presence of oxalate ions.

The most striking observation concerning stasis associated with VW production was that cross-septa were not observed. This phenomenon may be merely a reflection of a biochemical lesion far removed from cross-wall synthesis or may be a direct action of the virulence antigens themselves.

It is generally believed that enzyme induction, as well as induction of temperate phage and many bacteriocins, is an all-or-none effect. When maximal production of an induced entity by an otherwise homogenous population does not occur, it is usually because a portion of that population was not induced. This situation probably exists in virulent P. pestis induced to synthesize VW by removal of Ca^{++} from a medium containing adequate Mg^{++}. In this case, the elimination of increasing amounts of the "repressor", Ca^{++}, should permit induction and stasis of an increasing percentage of the virulent population. Similarly, stepwise addition of the "inducer",

Mg^{++}, to cultures containing low concentrations of Ca^{++} should again result in induction of VW and stasis in increasing portions of the virulent populations. Either the above hypothesis, or the suggestion that all the virulent cells are fully, partially, or noninduced, depending upon the concentration of Mg^{++} and Ca^{++}, would explain the data given in Figure 5. Further work is required to determine the correct alternative. It should be remembered that temperature is the primary factor controlling the expression, in vitro, of the phenomenon studied in these experiments; as shown by other workers, lysis, stasis, and VW production occur at 37°C, but not at room temperature.

It is significant that little or no VW was detected in cultures containing $\sim$0.0025 M Ca^{++}, regardless of the amount of the added Mg^{++}; as noted by Higuchi et al,[9] the above concentration of Ca^{++} is similar to that found in human blood. On the other hand, the optimal condition for VW production in synthetic media was 0.02 M Mg^{++} and no added Ca^{++}. These values are identical to those recorded for intracellular fluid by Kugelmass,[28] but are in contrast to those reported by Endres and Herget[29] in horse leukocytes. The latter workers detected 0.0017 M Ca^{++}, but employed an ashed preparation and thus determined bound Ca.

If the environmental conditions that influence VW production in vitro apply equally well in vivo, then one might expect little or no VW to be produced by cells in the vascular system and that optimal production would occur by bacteria residing in intracellular fluid. In the absence of experimental data, this proposal must be made with reservations, since its corollary states that cells in blood should divide whereas those within phagocytes should remain static. However, virulent cells do grow within monocytes[30] and give the morphological appearance of obtaining sufficient Ca^{++} (W.A. Janssen, unpublished observations). In addition,[31] only poor growth is observed within carefully drawn human or mouse sera; in this case the limiting metallic cation is Fe^{++} or Fe^{+++}. Another observation that must be explained is that virulent cells grown experimentally en masse in the guinea pig are rich in VW antigens.[32] Nevertheless, there is a distinct possibility that the virulence antigens are induced following phagocytosis; their function remains obscure.

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